STRUCTURAL ANALYSIS OF PROTEINS IN EXTREME SALINE ENVIRONMENTS

by

Dr. M. Shoham & Prof. J. L. Sussman

Weizmann Institute of Science

CONTRACT NUMBER DAJA45-86-C-0016

Final Report

March 17, 1986 - March 16, 1989

The Research reported in this document has been made possible through the support and sponsorship of the U.S. Government through its European Research Office of the U.S. Army. This report is intended only for the internal management use of the Contractor and the U.S. Government.

TABLE OF CONTENTS

OBJECTIVES

The objective of the proposed research plan was to elucidate the three-dimensional structure of 2Fe-2S ferredoxin from *halobacteria* of the Dead Sea and its comparison, to the available structure of a homologous 2Fe-2S ferredoxin from a nonhalophilic source, the algae *Spirulina platensis*. We wanted to understand what sort of adaptation, on the molecular level, is needed so that a protein can function in a hostile environment such as the Dead Sea.

BACKGROUND

Life is commonly regarded as a rather fragile phenomenon. However there are some organisms known to live in unusual environments like glaciers, geysers, salt lakes and deep sea, in which conditions are fatal to other organisms. In fact they represent the extreme limit in biological adaptation to harsh environments such as high temperature, high pressure and salt concentration (Jaenicke, 1981).

Many thermophilic organisms are known today, among them bacteria, algae and fungi. The group of true thermophiles, i.e. organisms that thrive in high temperatures and are not active at ambient temperature, include only a few species of eubacteria and most of the species now classified as archaebacteria. They are found in boiling springs and volcanic ponds (Brock, 1978). The absolute maximum temperature for growth of eukaryotic cells is around 62°C and for growth of photosynthetic algae and bacteria around 75°C (Brock, 1978).

Some organisms have been found to live in marine environment and in deep (up to 10Km below sea level) freshwater lakes in which hydrostatic pressure is nearly 1000 atm., they are known as barophilic organisms. In addition to high pressure, barophilic organisms may also be subject to wide variation of pressure, for example, Myctophids migrate daily 300 to 500 meter vertically which corresponds to 30-50 atm. changes. Some of them are known to live in high temperature (~300°C) as well as high pressure (~300 atm.) and therefore must adapt both to high pressure and temperature (Hochachka & Somero, 1973).

Halophilic organisms require high salt concentrations (1-4M) for their growth, stability and activity. They are found in salt lakes, e.g. the Great Salt Lake and the Dead Sea, and in salt sea muds. Halophilic and non-halophilic organisms are known to have similar overall metabolic pathways as well as very similar mechanism of DNA replication and protein synthesis (Jaenicke, 1981).

Different organisms have developed various mechanisms to protect themselves against harmful effects of high temperature, pressure and high salt concentration. We will concentrate on strategies to overcome possible harmful effects of high external salt concentration. Those strategies are correlated with the following phenomena: halo-adaptation and halo-tolerance, the first refer to halophilic organisms which are stable **only** in high salt concentration and therefore their cellular biochemical machinery has to adapt to it, whereas the later refers to non-halophilic organisms which are stable at low salt concentration however, if there is a drastic increase in salt concentration they can, by different mechanisms, stay stable and active. We will first discuss the mechanisms by which non-halophilic organisms can tolerate high salt and than halo-adaptation of halophilic organisms.

When non-halophilic organisms are faced with a drastic increase in extra cellular salt concentration and therefore increase in osmolarity, an appropriate adjustment in the internal osmotic concentration of the cell is needed, otherwise the cell would shrink. The internal excess solutes must not interfere with necessary metabolism and regulation. In many non-halophilic organisms the osmotic balance is achieved by increasing the intracellular amino acid concentrations, such as proline and glutamic acid, in the following way: after an osmotic shock and before the accumulation of amino acid, water is removed from the cell such that the cell volume will decrease. Once amino acid concentration has begun to increase within the cell, water is drawn back into the cell and therefore the cell volume is increased (Hochachka & Somero, 1973). Some other non-halophilic organisms selectively exclude Na^+ ions, allowing a moderate K^+ uptake maintaining near osmolarity by increasing intracellular concentration of polyols like glycerol, which is compatible solute to the external salt (Borowitzka & Brown, 1974; Gustafsson & Norkrans, 1976). Halo-tolerance in non-halophilic bacteria is correlated with the ability to accumulate amino acids or glycerol, these are not disruptive for normal metabolic activity or regulating mechanisms in the cell.

In contrast, in halophilic bacteria, osmotic equilibrium is achieved by increasing intracellular K^+ concentrations without increasing the concentration of polyols. The high internal salt concentrations thus generated might be expected to disrupt the activity and regulation of the bacterial enzymes. In such cases some adaptation, to high salt, of the structure and function of proteins becomes mandatory if they are to maintain their essential activity in the abnormal hyper saline environment. The adaptation of protein structure to high salt may become so drastic as to render the enzymes unstable or inactive at lower salt concentrations. The mechanism by which halophilic proteins are adapted to high internal concentrations of K^+ is not known. However, some observations regarding halophilic organisms properties, e.g. requirement for high salt concentration for their stability, excess of acidic over basic amino acid, and low frequency of hydrophobic amino acid, have suggested several alternative principal models:

a) Charge screening of the molecule induced by the excessive acidic amino acids (Baxter, 1959; Soo-Hoo & Brown 1967; Lanyi, 1974).
b) Stabilization of hydrophobic interactions at high salt concentration. The requirement for high salt concentration may reflect the lack of nonpolar residues and the requirement to increase the hydrophobic character, through a "salting-out" effect (Lanyi, 1974).
c) Maintenance of high hydrated protein surface by creating carboxyl clusters capable of binding highly ordered water (Werber *et al.*, 1978; Pundak & Eisenberg, 1981).
d) Halophilic proteins may be considered as large anions which effectively will compete for water of hydration with the high salt environment, and thus the relatively low hydrophobicity of its composition would allow the protein to remain in solution even at high salt concentration (Rao & Argos, 1981).

These different models may reflect the fact that the various groups used different proteins as their model for halophilic adaptation studies, and that actually no single mechanism explains all the observations.

One of the halophilic family is *Halobacteriaceae* which is subdivided into the genus *Halobacterium* containing the rod-shaped bacteria and the genus *Halococcus* containing the coccoid forms (Gibsons, 1974). A key difference between the two is their cell wall construction. *Halobacteria*, whatever their shape, have very thin outer envelopes that are easy to break mechanically, and most of them lyse in water. *Halococci* have thick cell envelopes that are difficult to break mechanically, they do not lyse in water and therefore survive exposure to much lower salt concentrations than *halobacteria*. Despite these differences, there are many similarities in biochemistry and physiology between these two genera, e.g. both require more than 2M NaCl in their media for growth, obtain energy from amino acids, grow in media with high concentrations of protein hydrolysates, have very high intracellular salt concentrations (potassium being a major ionic species), have proteins which are considerably more acidic than those found in non-halophilic organisms, have similar mole% (66-68%) of GC in their DNA which is higher than non-halophilic bacteria (Ginzburg *et al.*, 1978).

As was mentioned before, some of the halophilic organisms live in the Dead Sea. The Dead Sea is one of the most saline environments in the world. It contains about 322g/L of salts (Oren & Shilo, 1981). The major cations found there are (in g/L):Mg^{+2} 40.7, Na^{+} 39.2, Ca^{+2} 16.9, and K^{+} 7.3, while the major anions are Cl^{-} 212.4, and Br^{-} 5.1. Elazari-Volcani isolated several species of extreme halophilic microorganisms from the Dead Sea (Elazari-Volcani, 1940). One of them was *Halobacterium marismortui*, which has the ability to produce nitrous oxide and nitrogen gas from nitrates (Werber & Mevarech, 1978).

I. Halophilic Ferredoxin

In the course of evolution different organisms have adapted to different environments, occasionally to very harsh ones. It is of great interest to explore the limits of biological adaptation to extreme temperature, pressure or salinity. Some understanding can be gained from the study of organisms which live in such inhospitable ecological niches where life seems almost impossible. These organisms have evolved special adaptation mechanisms to survive where other forms of life cannot exist. In an attempt to understand the requirements for maintaining life at high salinity on the molecular level, we undertook the study of proteins from halobacteria which live in the saltiest body of water found on earth, the Dead Sea (Steinhorn & Gat, 1983). Halophilic proteins are active at intracellular supersaturated salt concentrations (Eisenberg & Wachtel, 1987; Werber, Sussman & Eisenberg, 1986), for example, the internal potassium concentration of *Halobacterium marismortui* is 4M (Werber *et al.*, 1978). "Normal" proteins in non-halophilic cells would be precipitated out and cease to function under these conditions. What are the structural requirements for a protein to remain soluble and active at practically saturated salt solutions?

As a model for the adaptation process of a protein to an extremely saline environment, we have investigated the crystal structure of a 2Fe-2S ferredoxin from the halophilic archaebacterium *Halobacterium marismortui* (HmFd). Ferredoxin represents a good choice for studying haloadaptation because it is ubiquitous from prokaryotes to higher plants and because the three-dimensional structure of non-halophilic ferredoxins with the same 2Fe-2S prosthetic group from the blue-green algae *Spirulina platensis* (SplFd) (K. Fukuyama *et al.*, 1980; Tsukihara *et al.* 1981) and from *Aphanothece sacrum* (AsFd) (Tsukihara, *et al.*, 1983) are known. Comparison of these ferredoxin structures highlights distinctive structural characteristics of the halophilic protein.

Some 50 2Fe-2S ferredoxins from different organisms have been sequenced (Tsukihara *et al.*, 1982; Protein Identification Resource (PIR) 1986). They fall into two major categories, those from plants, algae and non-halophilic bacteria having about 98 residues, and those from halobacteria with 128 amino acids.

HmFd, a halobacterial 2Fe-2S ferredoxin, resembles the plant ferredoxins except that it is longer, with a molecular mass of 14,000 daltons, and richer in negatively charged residues (Hase *et al.*, 1980; Kerscher *et al.*, 1976). HmFd has an extra 22-residue N-terminal and a 7-residue C-terminal region in comparison with its non-halophilic counterpart. Thirty four out of its 128 amino acid residues are either aspartic or glutamic acid, while on the other hand there are only six basic residues. The sequence alignment of HmFd (Hase *et al.*, 1980) and SplFd (Matsubara, Wada & Masaki, 1976; Wada *et al.*, 1974) reveals extensive homology in the core parts, particularly in the vicinity of the four cysteine residues that comprise the ligands for the iron-sulfur cluster (Fig. 1). The homology gets progressively weaker farther from the active site in the direction of both termini (Sussman, Brown & Shoham, 1986).

II. Halophilic Malate Dehydrogenase

Halophilic malate dehydrogenase (hMDH) is an enzyme from *Halobacterium marismortui*. Enzymes from extremely halophilic organisms have been shown to have different properties compared to non-halophilic enzymes e.g. requirement for high salt concentration for activity and stability (in such salt concentration the non-halophilic enzymes will be inactive), excess of acidic amino acid (Lanyi, 1974; Ginzburg, Sachs & Ginzburg, 1970; Bayley & Morton, 1978), yet no differences with respect to enzyme mechanism have been found. Therefore they are extremely fascinating enzymes to study with respect to the halo-adaptation phenomenon, hMDH being one of these enzymes. Biochemical and biophysical studies have shown hMDH to have some unusual properties with regard to halo-adaptation which can not be explained by current theories in the literature. Furthermore, the 3-D structure of a non-halophilic MDH from pig heart has already bean determined (Hill *et al.*, 1972). It catalyzes the following reaction:

$$\text{L-Malate} + NAD^+ \longleftrightarrow \text{Oxaloacetate} + NADH$$

The molecular weights of Porcine heart non-halophilic MDH from both cytoplasm and mitochondrial sources, were found to be 72,000-68,000 respectively (Tsernoglou, Hill, & Banaszak 1972). MDH has two subunits which have identical chemical structures, each containing one NAD binding site. The two subunits are related by an approximate two-fold axis of symmetry (Hill *et al.*, 1972).

hMDH is also composed of two identical subunits with total molar mass of 87,000 (per dimer). hMDH has a marked excess of acidic over basic amino acid residues, 18.8 mole% acidic residues (aspartate and glutamate) and 7.3 mole%basic residues (lysine and arginine) (Mevarech, Eisenberg & Neumann, 1977), whereas for non-halophilic MDH from cytoplasmic beef heart the values are 11.0% and 13.7% respectively (Siegel & Englrad, 1962). The difference between acidic and basic residues (in %mole) is 11.5 for hMDH and -2.7 for MDH so that hMDH has much higher excess of acidic amino acids. Halophilic enzymes are stable as long as the salt concentration is kept in a multimolar range, e.g. hMDH can be kept stable and active for several years in 4-4.3M NaCl, pH = 7, at room temperature (Mevarech, Eisenberg & Neumann, 1977; Mevarech & Neumann, 1977).

Eisenberg, Zaccai and collaborators have made an extensive study on stability, solvent interactions and activity of hMDH by using ultracentrifugation, small angle X-ray and neutron scattering techniques (Calmettes, Eisenberg, & Zaccai, 1987; Zaccai, Wachtel & Eisenberg, 1986; Eisenberg, & Wachtel, 1987; Zaccai, Bunick & Eisenberg, 1986; Zaccai *et al.* 1989). They contend that some of hMDH properties cannot be explained by using current models in literature.

Activity and stability measurements of hMDH were done in presence of very strong concentrations of different solvents, $MgCl_2$, NaCl, KCl, and K_2PO_4. $MgCl_2$ is a "salting-in" solvent, K_2PO_4 is a "salting-out" solvent and NaCl and KCl have neutral effects with respect to protein stabilization. "Salting-in" and "salting-out" phenomena occur at high salt concentration, however they are not well understood. It is believed that they are correlated with the nature of interaction between water-protein and water-salt. In the presence of certain salt the protein solubility increases when the possibilities for favorable protein-water interactions increases (compared to salt-water interactions)- this is known as the "salting-in" phenomenon. However if the salt will compete with each other and with the protein for water, dehydration of the protein molecule occurs therefore the protein precipitates- this is known as the "salting-out" phenomenon. A certain salt can be "salting-in"as well as "salting-out" salt depending on the nature of the protein.

Some of the unusual properties of hMDH based on Eisenberg and Zaccai studies (Calmettes, Eisenberg & Zaccai, 1987; Zaccai, Wachtel & Eisenberg, 1986; Eisenberg & Wachtel, 1987; Zaccai, Bunick & Eisenberg, 1986; Zaccai *et al.* 1989) are:

a) Although $MgCl_2$ is a "salting-in" salt, it stabilizes hMDH structure in a limited range of concentrations(bell-shaped curve), while in all the other solvents hMDH stability increases to a maximum.

b) hMDH associates with unusually high amounts of water and salt, 0.87g water/g protein and 0.35g NaCl/g protein (Pundak & Eisenberg, 1981; Zaccai, Wachtel & Eisenberg, 1986; Eisenberg, 1981). For comparison, the corresponding values for the non-halophilic protein e.g. bovine serum albumin (BSA), are 0.23g water/g protein and 0.012g NaCl/g protein. The high water-and salt-binding ability is lost when hMDH is denatured and unfolded at low salt concentration. However, in K_2PO_4, hMDH is hydrated to about 0.4g water/g protein and negligible amount of salt binding.

c) Using the neutron scattering contrast technique the radius of gyration of the protein itself and of the solvent could be determined by comparing the radius of gyration of the protein in presence of H_2O to that in presence of D_2O. (when D_2O is added to the protein exchange of the labile hydrogen atoms is expected). The radius of gyration of hMDH in all solvents were approximately the same and higher than non-halophilic MDH. The radius of gyration of the halophilic MDH itself is 27.9 Å, the associated water and salt distribution is 40 Å and that of the whole particle is 34.5 Å. For comparison the radius of gyration of the whole particle of non-halophilic MDH is 24.7Å.

To account for all the above results G. Zaccai, H. Eisenberg and their collaborators proposed a structural model for hMDH in solution shown in Fig 2.

According to this model hMDH has a protein core similar to non-halophilic MDH with about 20% of the protein extending out of the core (the loops in Fig. 2) and available for interaction with water and salt molecules. The weak hydrophobicity of the core is not strong enough to stabilize the active structure and therefore the hydration interaction must add to the stability. The protein cannot compete with the highly concentrated salt for water molecules therefore the protein evolved a quaternary structure that can coordinate hydrated salt anions and cations at higher local concentrations than the surrounding solvent. This structure binds large amounts of water and salt molecules. At low salt concentration the protein unfolds and looses its bound water and salt molecules because the chemical potential of the salt in the solvent is too low for the protein-water-salt complex to be formed. At sufficiently high salt concentration, as the solvent-salt concentration approaches that in the complex, the chemical potential of the water is too low, so that the water "leaves"the complex, thus destabilizing the protein structure. This is the case in $MgCl_2$ which is soluble beyond 1.6M and it's concentration in the complex is also 1.6M. To explain the "normal" hydration obtained for hMDH in high concentrations of "salting-out" K_2PO_4 they claim that the hydrophobicity of the protein core is sufficient to stabilize the active enzyme. It is clear that a more detailed structure of hMDH is needed in order to fully understand its properties.

RESULTS AND DISCUSSION

I. Halophilic Ferredoxin

Here we report the crystal structure of a 2Fe-2S ferredoxin from *Halobacterium marismortui* at 2.5Å resolution. The most striking feature of the structure is the presence of a spatially distinct domain made up of regions from the amino- and carboxy termini. A possible function of this domain maybe to solvate the protein in the intracellular supersaturated salt solution.

a) Structure Determination

HmFd was isolated and purified according to Werber & Mevarech (Werber and Mevarech, 1978) and crystallized from 3.8 M phosphate buffer at pH 7.0 as reported previously (Sussman *et al.*, 1979). Large hexagonal prisms, 1mm in cross section and 0.5mm in height, were grown by seeding over a period of several weeks. X-ray data were collected at room temperature on a Nonius CAD4 diffractometer from native crystals and one suitable heavy-atom derivative, potassium tetracyanoplatinate to 3.3Å resolution. Recently another native dataset to 2.0Å resolution was measured at -150°C on a Rigaku AFC5-R rotating anode diffractometer, using a cryogenic technique developed by Hakon Hope (Hope, 1985). Additional phasing was provided by the anomalous scattering of the iron-sulfur cluster of native protein crystals. An anomalous difference Patterson and a difference Fourier synthesis using the Single Isomorphous Replacement (SIR) phases were used to locate the iron-sulfur cluster. SIR phases were combined with anomalous scattering phase information, using a method developed by Hendrickson *et al.* (Hendrickson, Smith & Sheriff, 1985) and yielded an electron-density map at 3.3Å resolution. The envelope of the protein could readily be outlined on this map. The polypeptide backbone was traced from stacked transparent sheets of this electron-density map. A protein model incorporating the known amino-acid sequence was fitted to the electron-density map using the computer program FRODO (Jones, 1978) on a Vector General computer graphics display. We have done a preliminary refinement of the model by the reciprocal space least-squares techniques CORELS (Sussman *et al.*, 1977) and PROFFT (Hendrickson & Konnert, 1980; Finzel, 1987) to an R-factor of 0.27 at 2.5Å resolution. Parameters of the crystallographic structure determination and refinement are summarized in Table 1. A representative portion of the resultant electron-density map is shown in Fig. 3. Other crystallographic computing was done with the software packages ROCKS (Reeke, 1984) and PROTEIN (Steigemann, 1974).

Molecular replacement attempts using the known structure of SplFd and AsFd in reciprocal space (Crowther, 1972) were unsuccessful. However, the similarity between these two homologous structures and HmFd could clearly be demonstrated **in real space** by an unequivocal positioning of the α-carbon skeleton of

SplFd in the electron-density map at 3.3Å resolution. This was done by anchoring the 2Fe-2S cluster of the SplFd molecule onto the determined iron-sulfur position in the electron-density map and rotating the model about this position. The fact that a unique and clearly outstanding solution was found in this rotation search is a strong indication for the similarity of the two structures (Fig. 4).

b) Molecular Conformation of 2Fe-2S Ferredoxin

The core of the protein, residues 37-119, consists of a 10-stranded antiparallel ß-barrel with a short α-helix at residues 48-55 (Fig. 5a & 5c). The ß-strands wrap around the barrel roughly perpendicular to the barrel axis. At the bottom of the barrel there is an external loop containing three of the four cysteine residues that serve as ligands to the iron sulfur cluster in the active site. The fold of the polypeptide chain around the iron-sulfur cluster is similar to that of SplFd and so is the tetrahedral coordination of the cluster. Cysteines 63 and 68 are bonded to one iron atom and cysteines 71 and 102 to the other.

A separate smaller domain, consisting of chain segments from the N- and C-termini of the molecule (residues 1-30 and 120-128) is attached to the ß-barrel. The existence of such a domain extending out from the main body of the molecule is consistent with low angle X-ray scattering data of HmFd in solution (Eisenberg and Wachtel, 1987). It is rich in aspartic and glutamic acid residues and part of it forms an Ω-loop (Leszcynski and Rose, 1986). Residues 1-8 form an extended chain which packs against the wall of the ß-barrel. Residues 8 through 18 form an Ω-loop with a distance of 4.5Å between α-carbon atoms 8 and 18 at the neck of the loop. In addition to hydrogen bonds at the neck and side chain interactions in its interior, extra stabilization of the Ω-loop is provided by stacking interactions between tyrosines 8 and 19 whose rings are roughly parallel at a distance of 3.4Å. Tryptophan 16 is part of this loop and is located on the surface of the protein and points towards the iron-sulfur cluster. The distance from the plane of the indole ring to the proximal iron atom is about 8Å. It is conceivable that Trp 16 serves as a mediator in the electron transport to and from the iron-sulfur cluster, possibly via Cys 68 which is located in between. Such a mechanism is consistent with the finding that the fluorescence of one tryptophan moiety in HmFd is quenched by the iron atoms. This tryptophan is also present in 2Fe-2S ferredoxin from *Halobacterium halobium* (Hase *et al.* 1977). Aromatic residues have also been implicated in electron transfer of ferredoxin from *Peptococcus aerogenes* (Adman, Sieker & Jensen, 1972; Carter *et al.*, 1974).

c) Similarity to 2Fe-2S Ferredoxin from *Spirulina platensis*

The folding of the core part of the protein is rather similar to that of the related SplFd, including the active site fold (Fig. 5b). Conserved residues between these two proteins are mainly confined to the region in and around the active site (Fig. 1). Alignment of the sequence of 28 different 2Fe-2S ferredoxins indicates the same trend (Tsukihara *et al.*, 1982). Further away from the active site, the similarity breaks down. The common

fold of HmFd and SplFd is confined to residues 37-119. The rms deviation between corresponding α-carbon positions in this region is 3.0Å, a somewhat high value which indicates significant local differences, although the overall topology is clearly identical. One also has to keep in mind that both the SplFd and HmFd structures have been only partially refined. In the remainder of the polypeptide chain, residues 1-36 and 120-128, there is no relationship whatsoever between these two proteins. For example, residue 35 is a conserved glycine in all non-halophilic 2Fe-2S ferredoxins, whereas in HmFd it is an aspartic acid which forms a salt bridge with lysine 118.

d) Charge Distribution on the Surface of the Protein

The numerous carboxyl residues are distributed on the surface of the protein with concentrations in three regions: the extra domain, the hinge region in between the two domains (residues 31-36) and the solvent exposed wall of the ß-barrel. The latter forms an amphipathic ß structure in which some of the carboxyls appear at every other residue, i.e. Glu 77, Asp 79, Asp 81 and Asp 83 all pointing outward from the surface of the protein. Likewise, the single short α-helix has carboxyl groups at Glu 50 and Glu 53, both pointing roughly in the same direction. Interestingly, the active site region is entirely devoid of negative charges, although the iron-sulfur cluster is located close to the surface of the protein. Thus it would not be surprising if this region plays a role in a non-charged interaction with another protein.

c) Adaptation to High Salt

What does the adaptation to high salt environment involve? A possible mechanism of adaptation is the enhancement of the ability of the protein to bind sufficient amounts of water necessary for stability and function. Free water is scarce in the Dead Sea as well as inside the halobacterial cell. Therefore, halophilic proteins need residues capable of binding many water molecules. Consequently, a general characteristic of most of these proteins is the preponderance of aspartic and glutamic acid residues, which have the highest hydration capacity among the naturally occurring amino acids, with each carboxyl group capable of binding from 6-8 water molecules (Kuntz, 1971).

The adaptation of ferredoxin to high salinity might be achieved by the added extra domain. This domain, including the hinge region (1-36, 120-128), contains 36% acidic residues and may act as a water binding "handle". Indeed HmFd is soluble in very high ionic strength solutions. While the non-halophilic SplFd which is also quite acidic (~20%) was crystallized in 87% saturated ammonium sulfate (Tsukihara *et al.* 1981) the HmFd crystals dissolve in 95% saturated solution.

We postulate that the function of the extra domain may be to solvate the protein in the intracellular supersaturated salt solution. This carboxyl-rich domain may provide the necessary water-binding capacity to

allow for the solvation of the protein in an aqueous phase where free water is scarce in much the same way as a hydrophobic leader sequence causes solvation of a protein in a lipid bilayer.

Rao and Argos (Rao & Argos, 1981) made structural predictions about HmFd based on the sequence alignment with SplFd and its crystal structure. They assumed the two structures to have a common fold and correctly predicted most of the extra acidic residues in the core region to be located at the surface of the ß-barrel or the α-helix. They also suggested that the additional N- and C-terminal segments form a structural fold protecting the barrel face which is uncharged in SplFd. However, rather than shielding parts of the protein, the small domain appears to provide extra solvation capacity for the entire molecule. Rao and Argos predicted an internal salt bridge between Asp 29 and Lys 112 in HmFd. These residues are in fact very far apart (~27Å), however there is an internal salt bridge between Asp 36 and Lys 118. NMR measurements of HmFd in solution indicate the single histidine in the molecule, His 119, to be located on the surface of the protein (Gochin & Degani, 1985). The crystal structure proves this to be correct. His 119 serves as ligand to the tetracyanoplatinate derivative together with Met 30, Asp 17 and Ser 28.

In view of the many carboxyl groups on the surface of the protein it is extremely interesting to explore the water structure around the protein. With the recently measured 2Å low temperature (-150°C) X-ray data set it is likely that we shall be able to observe more than one hydration layer. At the current stage of refinement, 2.5Å resolution, 32 solvent molecules have been located (Table 1), and further refinement is in progress.

Does the solvation domain represent a general phenomenon of haloadaptation? Some halophilic proteins are larger than their non-halophilic counterparts of known three-dimensional structure: malate dehydrogenase from *Halobacterium marismortui* (hMDH) and from porcine heart have molecular weights of 87,000 and 72,000 daltons, respectively (Mevarech, Eisenberg & Neumann, 1977; Pundak & Eisenberg 1981; Birktoft *et al.*, 1982). Thus hMDH may possess an extra solvation domain while other halophilic proteins, which are no larger than their non-halophilic counterparts, like glutamate dehydrogenase or dihydrofolate reductase (Eisenberg & Tomkins, 1968), may lack it. The need for an extra solvation domain in HmFd may be a consequence of the covalently bound iron-sulfur cluster which confers extra rigidity on the molecule. In fact, removal of the iron-sulfur cluster causes the collapse of the structure into a random coil (Gochin & Degani, 1985). It is conceivable that in such a tightly restrained fold a more massive replacement of residues by aspartic or glutamic acid cannot be accommodated. Other proteins may be more flexible and may tolerate more replacements of non-acidic by acidic residues, thus having no need for an extra solvation domain. Nonetheless, the formation of a distinct solvation domain is apparently one way nature has chosen for the adaptation of a protein to extreme salinity.

II. Halophilic Malate Dehydrogenase

In the late 1970's M. Mevarech and E. Neumann, worked out a procedure to purify MDH from extremely halophilic bacteria of the Dead Sea (Mevarech, Eisenberg & Neumann 1977). The procedure includes: precipitation by ammonium sulfate, fractionation on sepharose-4D using a decreasing concentration gradient of ammonium sulfate, gel permeation chromatography on sephadex G-100, chromatography on hydroxylapatite and affinity chromatography on 8-(6-a,inohexyl)amino NAD^+ sepharose at 4.26M NaCl.

First attempts to crystallize hMDH were made by Prof. Ada Yonath several years ago. Very thin crystals were obtained by dialyzing hMDH against 2.9-3M phosphate buffer, pH = 7. The crystals did not survive under X-ray irradiation. Better crystals were then obtained by Dr. Michal Harel. The enzyme was dialyzed against 2M phosphate buffer, pH = 7, and crystallized by vapor diffusion using the hanging drops technique, against a reservoir solution of 2.6M phosphate, at room temperature. Large crystals up to 1mm in their longest dimension developed within several weeks. Preliminary X-ray studies on a Rigaku AFC5-R rotating anode diffractometer indicate the crystal to be monoclinic, space group I2, with a = 113.8Å, b = 122.8Å, c = 126.7Å, ß = 98.1° (Harel *et al.*, 1988). These crystals diffract to 2.5Å and survive for about 24 hours in the X-ray beam when cooled to ~0°C, and last about half that long at room temperature. Moreover, the crystals showed a tendency to cleave easily and were quite difficult to handle.

Our initial goal was to obtain hMDH crystals which would be stable under X-ray irradiation. Starting from an enzyme preparation that was dissolved in a solution of 4.2M NaCl, 50mM phosphate buffer, pH = 7, our first approach was to transfer the hMDH to phosphate buffer 2.5M, pH = 7 (by dialysis). Using vapor diffusion in the hanging drops method at room temperature, crystals were obtained under the conditions listed in Table 2.

Nice crystals with an average size of 0.4x0.4x0.2mm were obtained from the above four conditions, one of them is shown in Fig. 6. Under the microscope each crystal looked as if it were single, however, when one tries to mount the crystals it quickly becomes apparent that each crystals is composed of several plates one on top of the other that come a part, like a deck of cards, and therefore can not be used for X-ray studies.

Large crystals were obtained at room temperature using MPD (Fig. 7). In this case the hMDH was kept in its original solution i.e. 4.2M NaCl, phosphate buffer 5mM, pH = 7. The 7µl drop contained 10mg/ml hMDH, 1.8M NaCl, 58% MPD. The 1ml reservoir contained 60-70% MPD. Large crystal up to 1.0x1.0x0.4mm were obtained.

By using a precession camera we were able to get still photograph and several small angle precession photographs, however the resolution decreased drastically after several hours upon X-ray irradiation and therefore we were not able to determine cell constants and space group by using the precession photographs.

Preliminary X-ray studies on a Rigaku AFC5-R rotating anode diffractometer operated at 15kW, at room temperature indicate the crystal to be orthorhombic, space group $C222_1$ with cell constants a = 114.4Å, b = 131.7Å, c = 124.2Å. Once again after several hours the intensity dropped drastically. Using techniques of Hope (Hope, Frolow & Sussman, 1987; Hope, 1988), for low-temperature X-ray data collection (at 90K), we were able to preserve the lifetime of crystal practically indefinitely in the X-ray beam. According to this method the crystals are transformed from their mother liquor to a hydrocarbon environment, then mounted with a standard glass fiber, and flash cooled with boiling nitrogen stream on the diffractometer apparatus. This approach prevents freezing of the solvent in the crystal. In our case, we did not transfer the crystal to a coating solvent as the crystal is grown from high concentration (70%) of MPD which is an antifreeze agent, but mounted it directly from its hanging drop. The cell constants and space group, also determined on the rotating anode diffractometer at 90K, are a = 113.1Å, b = 122.6Å, c = 123.4Å and $C222_1$. The change in unit cell volume is ~8.5% which implies a possible phase transition or packing change. Preliminary results on the area detector in low temperature (90K)have shown the crystal to diffract to high resolution ~2.6Å, (Fig. 8).

From the knowledge of unit cell Volume (V), molecular weight of the protein (M_w), and number of asymmetric units per unit cell (N), the values of V_M can be obtained from:

$$V_M = V/(M_w \cdot N)$$

V_M = volume of asymmetric unit/molecular weight.
Using the molecular weight of hMDH dimer it will lead to $V_M = 2.5$, falling in the range obtained by Matthews (Matthews, 1968). Therefore there are two monomers per asymmetric unit that are possibly related by anon-crystallographic symmetry. In many protein crystal structures that have been determined this is a two-fold axis, which greatly aids in helping to solve the phase problem. The solvent volume can be calculated from:

$$V_{solv} = 1 - 1.23/V_M$$

This will lead to 50% solvent volume for hMDH grown from MPD as well as from phosphate buffer. The estimated number of reflections to be measured can be calculated from:

$$N = (4\pi V)/3\ d_m^3$$

d_m = interplanar spacing.
V = unit cell volume

The total number of reflections for d_m=3Å of hMDH is approximately 260,000, if we will divide it by the redundancy, 8, we will get the approximate number of unique reflections 32,500. In the diffractometer it will take ~40 days while in the area detector it might take ~7 days.

III. Cryogenic Temperature X-ray Data Collection

During this period, we have been able to show, that many crystals of biological macromolecules can be cooled to cryogenic temperatures, while preserving their crystallographic integrity (Hope, Frolow & Sussman, 1987; Hope, 1988). This new technique has proven to be a powerful tool for both the extension of the resolution as well as to significantly improve the quality of our X-ray data collection. We have applied this *cryogenic data collection* technique to our studies of the 2Fe-2S ferredoxin (HmFd) from *Halobacterium Marismortui* (Sussman, Harel & Shoham, 1988), as well as a series of different DNA duplexes and proteins (see below).

In order to use this new technique, we designed and assembled a low-temperature attachment for our Rigaku AFC5-R rotating anode diffractometer. This consisted of a basic Enraf-Nonius low temperature apparatus that was available, in our laboratory, for this purpose. The apparatus is capable of supplying a regulated stream of freshly boiled, cold N_2 gas. A combination of evacuated glass tubing and styrofoam was used to construct an outlet tube to provide a fixed position cold gas stream that would allow relatively free motion of the CHI circle. The apparatus used initially gave a temperature near 115 K. In its present form it gives a steady temperature of about 90 K at the crystal site, with a liquid N_2 consumption of 0.7 l/hr. This apparatus is now being used for nearly all macromolecular data collection in our laboratory.

The low-temperature crystal mounting technique consists of first coating the crystals with a viscous oil in the crystallization droplet, removing all the mother liquor solution covering it. A single crystal is then picked up with a thin glass fiber, and placed directly in the stream of cold N_2. In our laboratory, this low-temperature mounting procedure usually takes about 1/2 hour, which is comparable to the time required to mount a protein crystal, inside a capillary with its mother liquor, in a conventional manner.

A data set for the protein ferredoxin from *halobacteria* of the Dead Sea, was measured with the crystal cooled to 115 K. During data collection two monitor reflections were repeatedly measured for approximately 100 times over three days of data collection. Their scan intensities were about 10,000 and 7,000 counts and showed only statistical variations in their intensities. This is extraordinary for protein data, and would be excellent for high-precision small-molecule data collection. Here it must be emphasized that this stability could not have been attained without the low-temperature technique.

Three-dimensional X-ray diffraction data have been collected on this system for a series of different proteins and nucleic acids, in addition to the ferredoxin from the extreme *Halobacteria* of the Dead Sea (Sussman, Harel

& Shoham, 1988; Sussman *et al.*, 1987b) a DNA tridecamer d(CGCAGAATTCGCG) (Sussman *et al.*, 1987a; Joshua-Tor *et al.*, 1988) & octamer d(GGGCGCCC) (Rabinovich *et al.*, 1987), human carbomonoxy hemoglobin (Shaanan, 1987), colicin immunity protein (Shoham, 1987) and soy bean agglutinin (SBA) (Shaanan, 1987). In general the space group and unit cell parameters of the proteins and nucleic acids as determined at this cryogenic temperature are all within 1% of the parameters as measured at room temperature or 4° C.

Although most of the macromolecules that we have tested can be shock cooled and X-ray data can be collected on them, SBA is an example of a protein that just does not diffract at all once it is cooled. We are testing alternative methods of shock cooling protein crystals to try to overcome these problem cases.

CONCLUSIONS

The work carried out during the three years of this project has justified the rationale which served as its basis. Thus the choice of proteins, namely ferredoxin, has permitted us to obtain the first three-dimensional structure of a halophilic protein at atomic resolution. This has led to the concept of a *solvent domain* to help explain the halophilic nature of this protein. Work on the study of the three-dimensional structures of extreme halophilic proteins has continued at the molecular level. During this period we have a) applied a new *cryogenic temperature data collection*, that we helped to develop, to extend the resolution and improve the quality of our X-ray data for the 2Fe-2S ferredoxin (HmFd) from *Halobacterium Marismortui* and b) continued our three-dimensional structural studies of the first halophilic enzyme, i.e. malate dehydrogenase (hMDH) also from *Halobacterium Marismortui*.

REFERENCES

Adman, E.T., Sieker, L.C. and Jensen, L.H. (1972) *J. Biol. Chem.* **248** 3987.

Baxter, R.M. (1959) *Can. J. Microbiol.* **5**, 47.

Bayley, S.T. and Morton, A. (1978) *CRC Crit. Rev. Microbiol.* **6**, 151.

Birktoft, J.J., Fernley, R.T., Bradshaw, R.A. and Banaszak, L.J. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 6166.

Borowitzka, L.J. and Brown, A.D. (1974) *Arch. Microbiol.* **96**, 37.

Brock, T.D. (1978) *in Thermophilic microorganisms and life at high temperatures*. New York/ Heidelberg/ Berlin: Springer.

Calmettes, P., Eisenberg, H. and Zaccai, G. (1987) *Biophysical Chemistry* **26**, 279.

Carter, C.W. Jr., Kraut, J., Freer, S.T. and Alden, R.A. (1974) *J. Biol. Chem.* **249**, 6339.

Crowther, R.A. (1972) *in The Molecular Replacement Method*, (Int. Sci. Rev. Ser., 13:) (Rossmann, M.G., Ed.) Gordon and Breach, New York, p.10.

Eisenberg, H. and Wachtel, E.J. (1987) *Ann. Rev. Biophys. Chem.* **16**, 69.

Eisenberg, H. (1981) *Quart. Rev. Biophys.* **14**, 141.

Eisenberg, H. and Tomkins, G.M. (1968) *J. Mol. Biol.* **31** 37.

Elazari-Volcani, B. (1940) Ph.D Thesis (in Hebrew) Hebrew University, Jerusalem.

Finzel, B.C. (1987) *J. Appl. Cryst.* **20**, 53.

Fukuyama, K., Hase, T., Matsumoto, S., Tsukihara, T., Katsube, Y., Tanaka, N., Kakudo, M., Wada K. and. Matsubara, H. (1980) *Nature* **286**, 522.

Gibsons, N.E. (1974) *in Bergey's Manual of Determinative Bacteriology, 8th Edition*, (Buchana, R.E. and Gibsons, N.E. eds.), Williams and Wilkins, Baltimore.

Ginzburg, M., Lepkipser, B., Porath, A. and Ginzburg, B.Z.(1978) *Energetics and Structure of Halophilic Microorganisms*. Elsevier-North Holland Biomedical press.

Ginzburg, M., Sachs, L. and Ginzburg, B.Z. (1970) *J. Gen. Phisiol.* **55**, 187.

Gochin, M. and Degani, H. (1985) *J. Inorg. Biochem.* **25** 151.

Gustafsson, L. and Norkrans, B. (1976) *Arch. Microbiol.* **110**, 177.

Harel, M., Shoham, M., Frolow, F., Eisenberg, H., Mevarech, M., Yonath, A. and Sussman J.L. (1988) *J. Mol. Biol.* **200**, 609.

Hase, T. *et al.* (1977) *FEBS Letters* **77** 308.

Hase, T., Wakabayashi, S., Matsubara, H., Mevarech, M. and Werber, M.M. (1980) *Biochim. et Biophys. Acta* **623**, 139.

Hendrickson, W.A. and Konnert, J.H. (1980) *in Computing in Crystallography* (Diamond, R., Ramaseshan, S., Venkatesan, K., Eds.) Indian Academy of Sciences, Bangalore, pp. 13.01-13.25.

Hendrickson, W.A., Smith, J.L. and Sheriff, S. (1985) *Methods in Enzymology* **115**, 41.

Hill, E., Tsernoglou, D., Webb, L. and Banaszak, L.J. (1972) *J. Mol. Biol.* **72**, 577.

Hochachka, P.W. and Somero, G.N. (1973) *Strategy of Biochemical Adaptation*. W.B. Saunders, Philadelphia.

Hope, H. (1985). *in Amer. Cryst. Assoc., Abstracts Ser. 2*, **13**, abstract PA3.

Hope, H. (1988) *Acta. Cryst.* **B44**, 22.

Hope, H., Frolow, F. and Sussman, J.L. (1987) *The Rigaku Journal* **4**, 3.

Jaenicke, R. (1981) *Annu. Rev. of Biophys. and Bioeng.* **10**, 1.

Joshua-Tor, L., Rabinovich, D., Hope. H., Frolow, F., Appella, E. and Sussman, J.L. (1988) *Nature* **334**, 82.

Jones, A. (1978) *J.Appl.Cryst.* **11**, 268.

Kerscher, L., Oesterhelt, D., Cammack, R., Hall, D.O. (1976) *Eur. J. Biochem.* **71** 101.

Kuntz, I.D. (1971) *J. Am. Chem. Soc.* **93**, 514.

Lanyi, J.K. (1974) *Bacteriol. Rev.* **38**, 272.

Leszcynski, J.F. and Rose, G.D. (1986) *Science* **234** 849.

Matsubara, H., Wada, K. and Masaki, R. (1976) *in Iron and Copper Proteins* (Yasunobu, K.T., Mower, H.F. & Hayaishi, O., eds.) Plenum Press, New York & London, pp. 1-15.

Matthews, B.W. (1968) *J. Mol. Biol.* **33**, 491.

Mevarech, M., Eisenberg, H. and Neumann, E. (1977) *Biochemistry* **16**, 3781.

Mevarech, M. and Neumann, E. (1977) *Biochemistry* **16**, 3786.

Oren, A. and Shilo, M. (1981) *Arch. Microbiol.* **130**, 185.

Protein Identification Resource (1986) "(PIR) Sequence Data Bank", The National Biomedical Research Foundation, Washington, D.C., release 10.0.

Pundak, S. and Eisenberg, H. (1981) *Eur. J. Biochem.* **118**, 463.

Rao, J.K.M. and Argos, P. (1981) *Biochemistry* **20**, 6536.

Rabinovich, D., Eisenstein, M., Haran, T.E. & Shakked, Z. (1987) IX International Congress of Biophysics, Jerusalem, abstract p. 113.

Reeke, G.N. Jr. (1984) *J. Appl. Cryst.* **17** 125.

Siegel, L. and Englrad, S. (1962) *Biochem. Biophys. Acta* **64**, 101.

Shaanan, B. (1987) unpublished results.

Shoham, M. (1987) IXth International Congress of Biophysics, Jerusalem, abstract p. 115.

Soo-Hoo, T.S. and Brown, A.D. (1967) *Biochem. Biophys. Acta* **135**, 164.

Steigemann, W. (1974) Ph.D. thesis, Technische Universitaet Muenchen.

Steinhorn, I. and Gat, J.R. (1983) *Scientific American* **249** 102.

Sussman, J.L., Brown, J. and Shoham, M. (1986) *in Iron-Sulfur Protein Research* (Matsubara, H., Katsube, Y., Wada, K. eds.) Japan Sci. Soc. Press, Tokyo/Springer-Verlag, Berlin, pp. 69-82.

Sussman, J.L., Harel, M. and Shoham, M. (1988) *in Computer-Assisted Modeling of Receptor-Ligand Interactions Theoretical Aspects and Applications to Drug Design* (Rein, R. & Golombek, A. Eds.) 171, A. R. Liss, Inc. N.Y.

Sussman, J.L., Holbrook, S.R., Church, G.M., Kim, S.-H. (1977) *Acta Cryst.* **A80**, 800.

Sussman, J.L., Joshua-Tor, L., Hirshberg, M., Saper, M.A., Frolow, F., Hope, H. and Appella, E. (1987a) *in Molecular Structure: Chemical Reactivity and Biological Activity*, (Stezowski, J.J, Huang, J.-L. & Shao, M.-C., eds.), Oxford University Press, Oxford pp. 195-200.

Sussman, J. L., Shoham, M., Harel, M, Frolow, F. and Hope, H. (1987b) International Meeting on Biochemistry, Beijing, abstract. B-A1-0435, p. 186.

Sussman, J.L., Zipori, P., Harel, M., Yonath, A. & Werber, M.M. (1979). *J. Mol. Biol.* **134**, 375.

Shoham, M., Sussman, J. L. & Harel, M. (1987). IXth International Congress of Biophysics, Jerusalem, abstract p. 26.

Tsernoglou, D., Hill, E. and Banaszak, L.J. (1972) *J. Mol. Biol.* **69**, 75.

Tsukihara, T., Fukuyama, K., Nakamura, M., Katsube, Y., Tanaka, N., Kakudo, M., Wada, K., Hase, T. and Matsubara, H. (1981) *J.Biochem.(Tokyo)* **90**, 1763.

Tsukihara, T., Kobayashi, M., Nakamura, M., Katsube, Y., Fukuyama, K., Hase, T., Wada, K. and Matsubara, H. (1982) *BioSystems* **15**, 243.

Tsukihara, T., Mizushima, M., Fukuyama, K. and Katsube, Y. (1983) in *Int. Summer School on Crystallographic Computing II*, Kyoto, Japan, pp. 63-64.

Wada, K., Hase, T., Tokunaga, H. and Matsubara, H. (1974) *FEBS Lett.* **55** 102.

Werber, M.M., Mevarech, M., Leicht, W. and Eisenberg, H. (1978) *in Energetics and Structure of Halophilic Microorganisms* (Caplan, S.R. and Ginzburg, M., Eds.), Elsevier, Amsterdam.

Werber, M.M. and Mevarech, M. (1978) *Arch. Biochem. Biophy.* **186**, 60.

Werber, M.M., Sussman J.L. and Eisenberg, H (1986) *FEMS Microbiol. Rev.* **39**, 129.

Zaccai, G., Bunick, G.J. and Eisenberg, H. (1986) *J. Mol. Biol.* **192**, 155.

Zaccai, G., Cendrin, F., Haik, Y., Borochov, N. and Eisenberg, H. (1989) *J. Mol. Biol.* in press.

Zaccai, G., Wachtel, E. and Eisenberg, H. (1986) *J. Mol. Biol.* **190**, 97.

Published Papers Based on This Research during this Period

1. H. Hope, F. Frolow & J. L. Sussman (1987) "Macromolecular X-ray data Collection on a Rotating Anode Diffractometer". The Rigaku Journal 4, 3-10 (1987).

2. M. Harel, M. Shoham, F. Frolow, H. Eisenberg, A. Yonath & J. L. Sussman (1988). "Crystallization of Malate Dehydrogenase from the Extreme Halophile Halobacterium Marismortui". J. Mol. Biol. 200, 609-610.

3. M. Hirshberg, R. Sharon & J. L. Sussman (1988) "A Kinked Model for the Solution Structure of DNA Tridecamers with Inserted Adenosines: Energy Minimization and Molecular Dynamics". J. of Biomolecular Structure & Dynamics. 5, 965-979.

4. J. L. Sussman, L. Joshua-Tor, M. Hirshberg, M. A. Saper, F. Frolow, H. Hope & E. Appella (1987). "A Kinked Model of a DNA Tridecamer with an Unpaired Adenosine: Energy Minimization and X-ray Structural Studies". in "Molecular Structure: Chemical Reactivity and Biological Activity", (eds. Stezowski, J.J, Huang, J.-L. & Shao, M.-C.), 195-200, Oxford University Press, Oxford.

5. L. Joshua-Tor, D. Rabinovich, H. Hope, F. Frolow, E. Appella & J. L. Sussman (1988). "The Three-Dimensional Structure of a DNA Duplex Containing Looped-out Bases". Nature 334, 82-84.

6. J. L. Sussman, M. Shoham & M. Harel (1989). "Protein Adaptation to Extreme Salinity: The Crystal Structure of 2Fe-2S Ferredoxin from *Halobacterium Marismortui*". in "Computer-Assisted Modeling of Receptor-Ligand Interactions: Theoretical Aspects and Applications to Drug Design", (R. Rein & A. Golombek, eds.) pp 171-187, Alan R. Liss, Inc, NY.

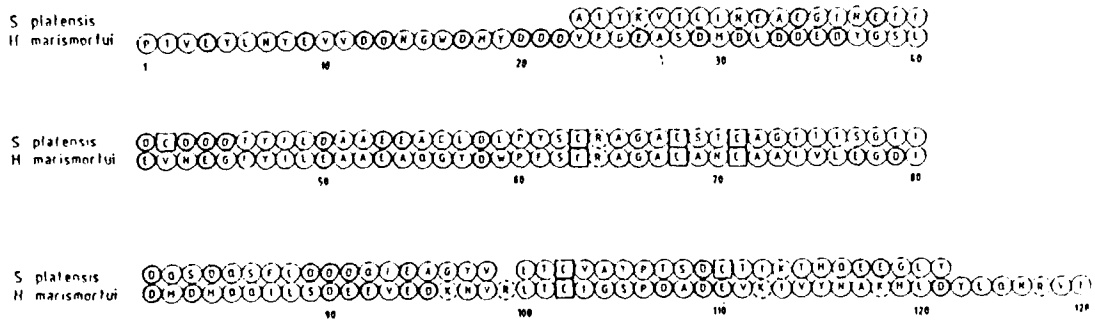

Figure 1. Amino acid sequence alignment of 2Fe-2S ferredoxins from a halophilic (*Halobacterium marismortui*) and a non-halophilic (*Spirulina platensis*) organism.

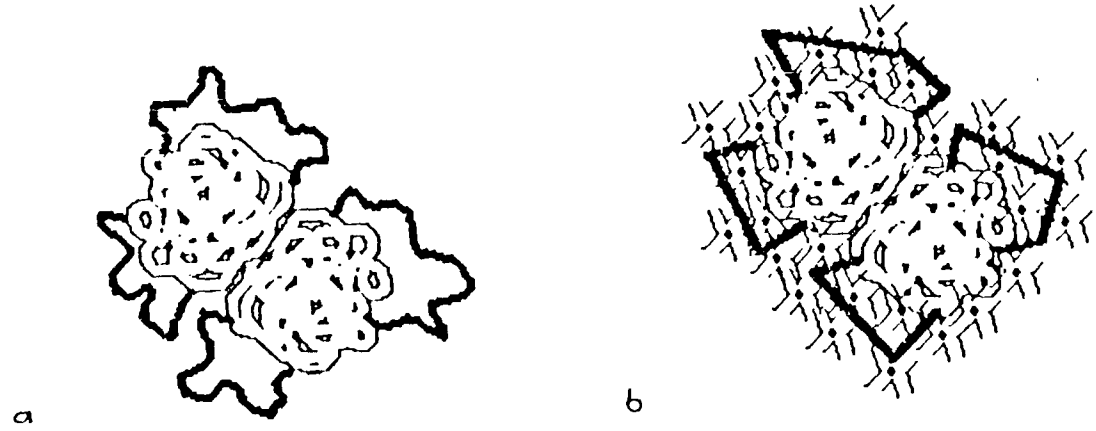

Figure 2. Schematic representation of hMDH solution structure (a) in K_2PO_4 (b) in KCl, NaCl, and $MgCl_2$.

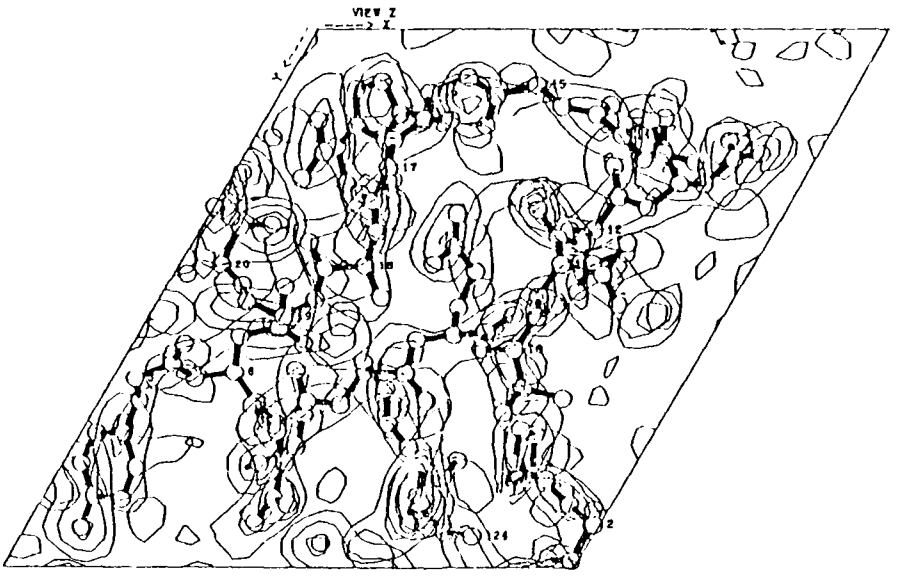

Figure 3. A 7Å thick portion of the 2Fo-Fc electron-density map at 2.5Å resolution of ferredoxin from *Halobacterium marismortui* as viewed down the unique z-axis. A ball and stick model of the corresponding part of the N-terminal solvation domain is superimposed on the map.

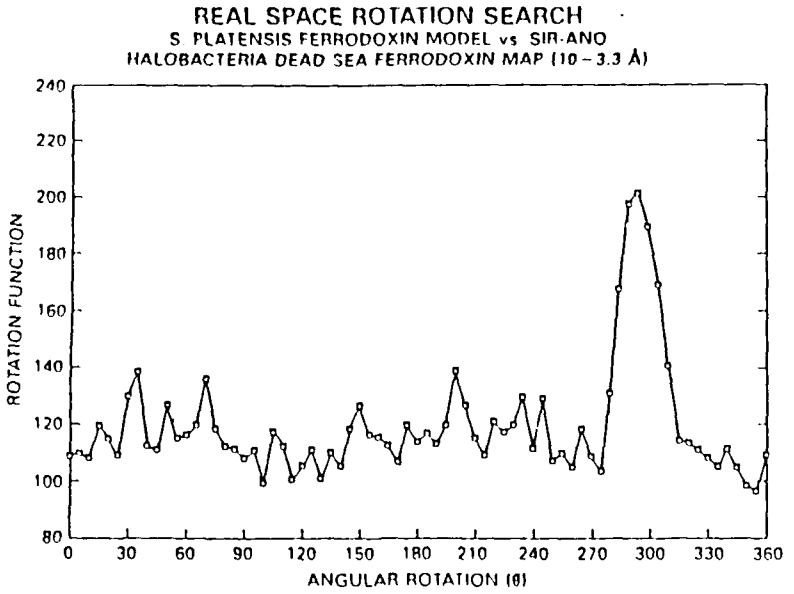

Figure 4. Real space rotation search (Steigemann, 1974) of the *Spirulina platensis* ferredoxin model on the *Halobacterium marismortui* 3.3Å resolution electron-density map. The map was calculated with phases based on a single isomorphous derivative (SIR), combined with anomalous scattering phases (ANO) inherent in the 2Fe-2S cluster. The highest peak in the rotation search in the θ1,θ2 plane is plotted as a function of the third rotation angle θ3. The single solution is an indication for the structural similarity of the two proteins.

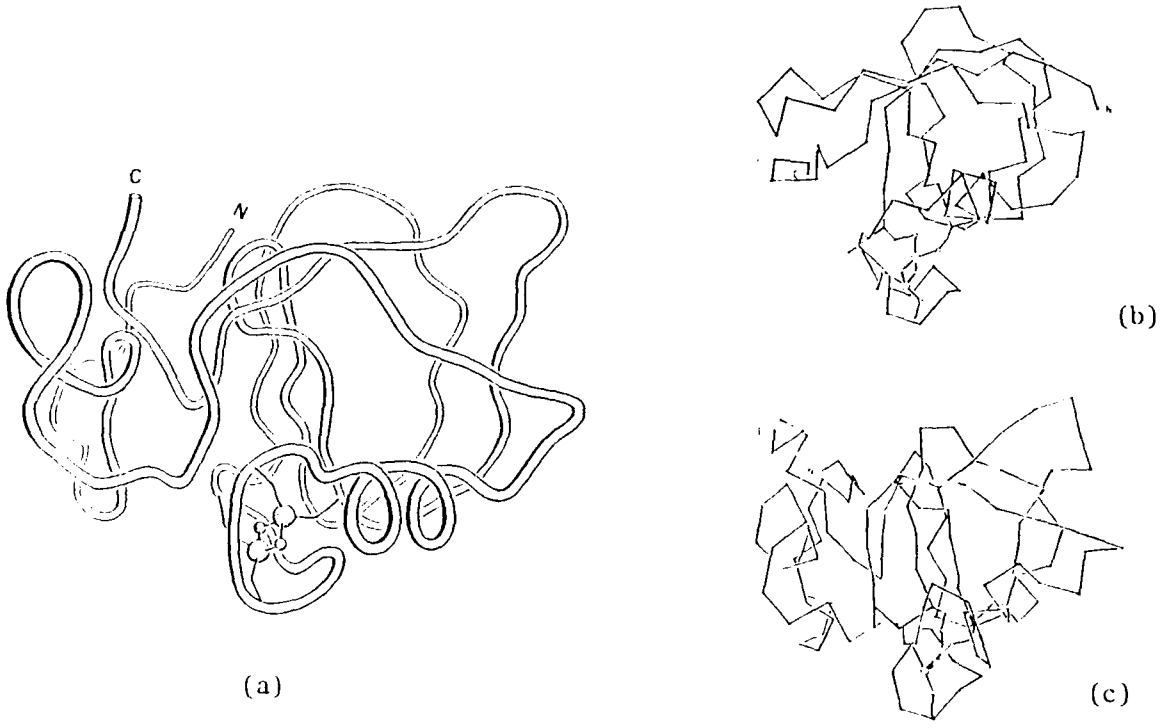

Figure 5. The fold of 2Fe-2S ferredoxin from *Halobacterium marismortui*. **(a)** Tube representation of the polypeptide chain of 2Fe-2S ferredoxin from *Halobacterium marismortui*. The 2Fe-2S cluster is shown as circles and its bonds to cysteines 63, 68, 71 and 102 as solid lines. Residues 1-30 and 120-128, shown on the left-hand side of the diagram, form a distinct domain, rich in Asp and Glu, whose apparent function is to solvate the protein in concentrated salt solution. **(b)** Drawing of the α-carbon positions of 2Fe-2S ferredoxin from *Spirulina platensis* (Tsukihara *et al.* 1981). The depicted orientation yields a maximum overlap of residues 37-119 in the two ferredoxin molecules. **(c)** Drawing of the α-carbon positions of 2Fe-2S ferredoxin from *Halobacterium marismortui*. The iron sulfur cluster atoms are drawn as larger circles.

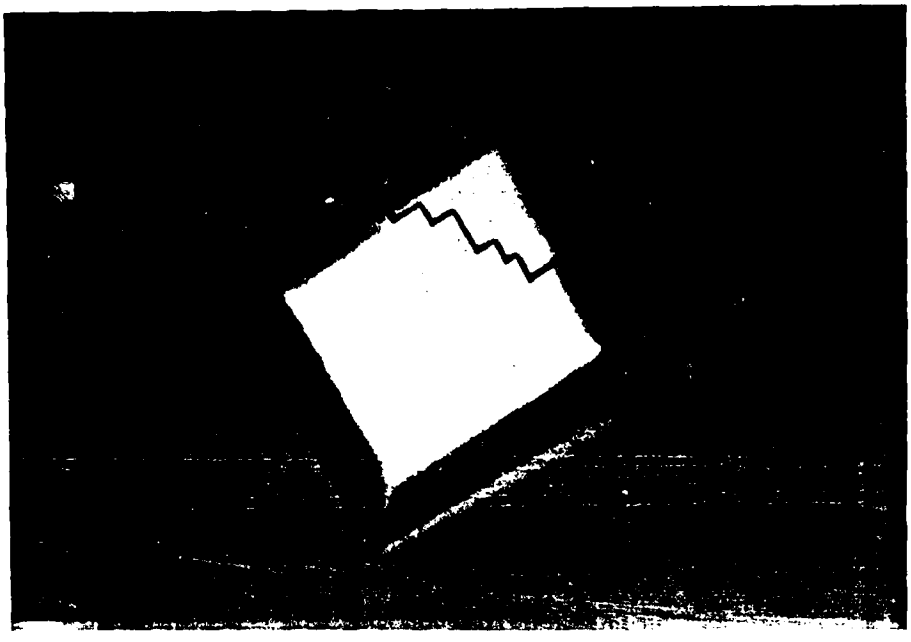

Figure 6. hMDH crystals grown in presence of 1M KCl, 0.36x0.36x0.1mm.

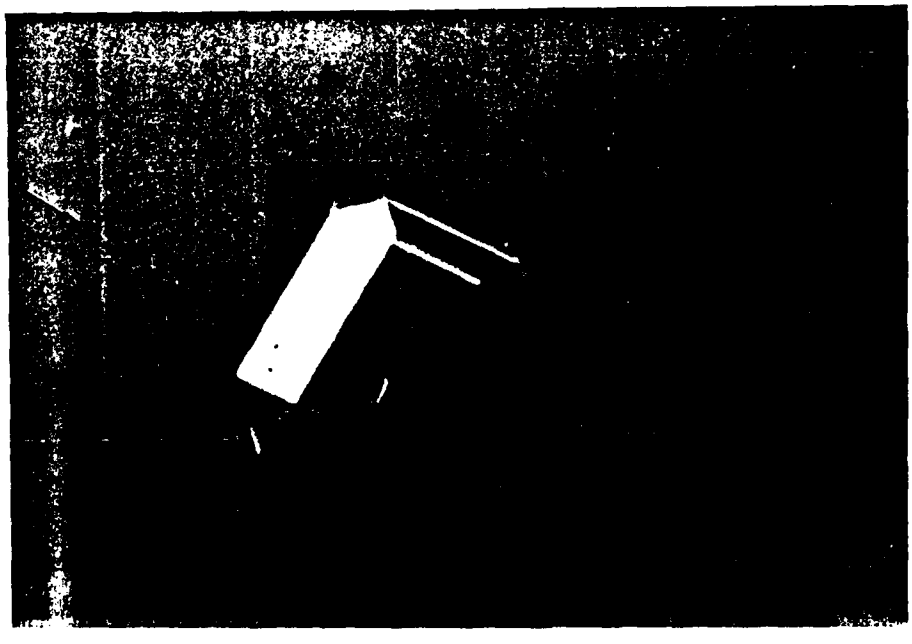

Figure 7. hMDH crystals grown in 65% MPD, 0.76x0.56x0.2mm.

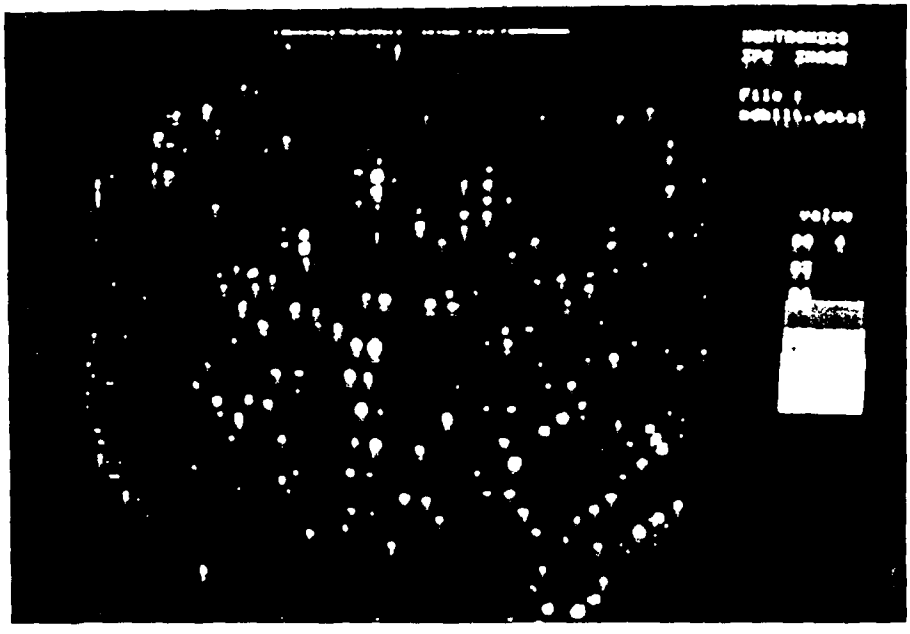

Figure 8. Diffraction photograph of hMDH crystal on area detector, 0.25° oscillation.

Table 1.Statistics for the structure determination of 2Fe-2S Ferredoxin from *Halobacterium marismortui*: phase determination and refinement.

Single Isomorphous Replacement						
Heavy atom	Soaking time	Reso-lution	No. of unique reflections	R_{iso}	No. of sites	R_{cen}
$K_2Pt(CN)_4$	3 days	3.3Å	2127	.13	3	.46

Anomalous Scattering of native Crystals & Phase Combination				
R_{ano}	Number of Acentric Reflections	FOM_{ANO}	FOM_{SIR}	FOM_{COMB}
.04	1513	0.25	0.43	0.52

Refinement at 2.5Å	
R = ΣFo-Fc/ΣFo	0.274
No. of reflections (6.0 to 2.5Å)	4483 (>3σ)
No. of atoms	1011
No. of Solvent atoms	32
No. of parameters (including individual B's)	4173
Average Fo	26.2 e
Average Fo-Fc	6.5 e
Average B factor	11.9Å^2
rms deviation bond length	0.04Å (0.030)*
rms deviation bond angle distance	0.09Å (0.040)
rms deviation from planar group	0.10Å (0.050)
rms deviation from trans peptide	5.0° (10.0)

Table 1. Statistics for the structure determination of 2Fe-2S Ferredoxin from *Halobacterium marismortui*: phase determination and refinement (*continued*).

FOM_{ANO} - Figure of merit due to anomalous scattering of native crystals containing Fe and S atoms.

FOM_{SIR} - Figure of merit due to single heavy atom derivative.

FOM_{COMB} - Combined figure of merit of ANO and SIR.

$$R_{ISO} = \frac{\sum_{h} ||F_{PH}obs| - |F_{P}obs||}{\sum_{h} |F_{P}obs|}$$

$$R_{CEN} = \frac{\sum_{h\ centric} ||F_{PH}obs| - |F_{P}obs + F_{H}calc||}{\sum_{h\ centric} ||F_{PH}obs| - |F_{P}obs||}$$

$$R_{ANO} = \frac{\sum_{h\ acentric} ||F_{hkl}| - |F_{\bar{h}\bar{k}\bar{l}}||}{\sum_{h\ acentric} (|F_{hkl}| + |F_{\bar{h}\bar{k}\bar{l}}|)/2}$$

F_P - Native protein structure factor.

F_{PH} - Heavy atom derivative structure factor.

obs - Observed.

calc - Calculated.

* - The numbers in parentheses represent the values to which these parameters were restrained.

Table 2: Conditions for crystallization.

Drop[1]	Reservoir[2]
hMDH 11mg/ml in Pi[3] 2.5M pH=7	Pi 2.7M pH=7, KCl 1M
hMDH 11mg/ml in Pi 2.5M pH=7	Pi 2.7M pH=7, NH_4Cl 1M
hMDH 9.4mg/ml in Pi 2.5M pH=7 and Ac[4]0.3M	Pi 2.3M pH=7, Ac 0.3M, MPD[5] 7%
hMDH 11mg/ml in Pi 2.5M pH=7	Pi 2.8M pH=7, 6% PEG[6]

(1) drop contain 6µl, (2) reservoir contain 1ml, (3) phosphate buffer, (4) Acetate buffer, (5) 2-methyl-2,4-pentadiol, (6) polyethylenglycol MW 3500.